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Rapport de mission

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Pathogénicité de la souche Gardel chez la souris

Des cellules BUEC (passage 30) ont été infectées *in vitro* avec la souche Gardel de *Cowdria* (passage 80). Lorsque 70 à 90 % des cellules sont lysées par la rickettsie, le surnageant est récolté et cfg à 200 X g pendant 5 min. afin de culoter les débris cellulaires. Le surnageant est ensuite cfg à 10000 X g pdt 20 min. Le culot est resuspendu dans du SPG (1 ml de surnageant infectieux est resuspendu dans 150 µl de SPG). Trois souris (NMRI) ont été infectées avec ce matériel (150 µl de SPG + *Cowdria* , par souris, par voie intraveineuse). Des souris inoculées avec du SPG sans *Cowdria* servent de témoin négatif. Aucunes des souris n'a présenté les symptômes de la maladie et toutes ont succombé à une infection, réalisée 1 mois après, avec le stock *Welgevonden* .

Au cours d'une seconde expérience, *Cowdria* (Gardel) a été préparée exactement de la même manière que précédemment. Des souris (NMRI) ont été infectées par voie intraveineuse mais aussi par voie intrapéritonéale (4 souris par groupe). De plus, un groupe de souris, infecté avec le stock Welgevonden de *Cowdria* (préparé de la même manière que Gardel), sert de contrôle positif. L'expérience est actuellement en cours.

Effet de l'IFNγ bovin recombinant sur le développement de *Cowdria* (Gardel) *in vitro*

L'effet inhibiteur de l'IFNγ bovin recombinant sur le développement de *Cowdria* (Gardel), *in vitro* dans des cellules endothéliales de jugulaires de chèvres (CJE107), a été confirmé. De plus, l'IFNγ est également capable d'inhiber complêtement le développement de *Cowdria* (Gardel) dans les BUEC. Enfin, le développement du stock Sénégal de *Cowdria* dans les CJE107 est également efficacement inhibé par l'IFNγ. L'effet de l'IFNα et du TNFα sur le développement de Gardel, *in vitro* dans diiférents types de cellules endothéliales est en cours à l'Université Libre de Bruxelles.

Les résultats concernant l'effet inhibiteur de l'IFNγ sur le développement de *Cowdria* feront l'objet d'une publication. Un manuscrit (v. annexe) intitulé "Inhibitory Effect of Recombinant Bovine Interferon Gamma on the Growth of *Cowdria ruminantium* in Bovine Endothelial Cells In Vitro" , est en

préparation et sera soumis à publication dans la revue "Infection and Immunity".

Mise au point d'un test de titrage de l'activité antivirale des IFNs α et γ de chèvre

Le test classique de réduction de l'effet cytopathogène du VSV (virus de la stomatite vésiculeuse) sur cellules bovines, qui permet de doser les IFNs bovins est inutilisable dans le cas des IFNs de chèvre (étant donné la spécificité d'espèce de ces derniers). La mise au point d'un test de réduction de l'effet cytopathogène du VSV sur cellules de chèvres permettrait de résoudre ce problême. Le VSV, produit sur cellules bovines, s'est révélé non infectieux pour des cellules endothéliales de chèvres (CJE107). L'adaptation du VSV aux CJE (par passage succéssifs à de faibles doses afin de limiter la production de particules interférentes) est en cours. L'utilisation d'autres types de cellules de chèvres (fibroblastes et cellules épithéliales de rein) sera également envisagée.

Production de *Cowdria* (Gardel) dans des BUEC cultivées en bioréacteur

Nous avons montré qu'il était possible de cultiver des cellules endothéliales sur microporteurs (billes de collagène) et d'obtenir ainsi une biomasse importante par culture en spinner (100 ml). Des essais en boîte de pétri ont montré que des cellules endothéliales bovines (BUEC) cultivées sur microporteurs conviennent parfaitement à la production de *Cowdria* (Sénégal) *in vitro* . Nous avons, au cours de ce séjour, fait un essai de production de *Cowdria* (Gardel) en bioréacteur (1litre).

Des BUEC ont été cultivées en spinner (100 ml) sur des microporteurs de type Cytodex III (3 mg/ml). Après une période de croissance de $\pm$ 10 jours les microporteurs sont entièrement colonisés par les cellules. Les 100 ml de milieu contenant les cellules sur billes ont été ajoutés à 900 ml de milieu (DMEM + sérum, antibiotiques, L-Glutamine et hepes) frais contenant 3 mg/ml de billes sans cellules. Cette expérience à été réalisée dans un bioréacteur de type Chemap (version 2,20). Le milieu n'est pas agité pendant 24 h. afin de favoriser l'ensemencement des billes par les cellules. Ensuite le mileu est agité à 40 rpm avec des pointes à 100 rpm pendant 20 sec. tous les jours

(pH:7,0 - 7,2 ; T°: 37°C ; O2: 21%). Un tiers du milieu est renouvelé tous les jours. Après 12 jours, 80 % des billes sont colonisées par les BUEC avec 20 à 100 % de colonisation par bille. A ce moment, le mileu est entièrement remplacé par du milieu d'infection (GMEM + sérum, antibiotiques, L-Glutamine, hepes et bouillon de Tryptose). 20 ml de surnageant provenant d'une boîte de 75 cm^2 présentant 80 - 90 % de lyse des BUEC dûe à *Cowdria* (Gardel passage 82) sont ajoutés au bioréacteur. Le milieu est entièrement renouvelé 3 jours après l'infection. Au jour 5 après l'infection, la quasi totalité des cellules étaient lysées mais sans qu'il y ait de colonies de *Cowdria* visibles dans le cytoplasme des cellules restantes. De plus, le milieu ne s'est pas révélé infectieux pour des BUEC cultivées en boîte de culture. La lyse des cellules dans le bioréacteur n'est donc pas dûe à *Cowdria* . Il est possible que les BUEC utilisées étaient trop vieilles (passage 30) ou qu'elles n'aient pas supporté le changement de milieu. Un autre essai sera réalisé avec des BUEC à passage 15 et en remplaçant de manière progressive le milieu de culture par le milieu d'infection.

Préparation du matériel pour le projet à l'ILRAD

Six boîtes (75 cm^2) de BUEC (passage 30) ont été infectées avec la souche Gardel de *Cowdria* (passage 86). Le surnageant, récolté lorsque ± 80 % des cellules sont lysées par la rickettsie (de nombreuses colonies de *Cowdria* sont visibles dans le cytoplasme des cellules), est cfg à 200 X g pendant 10 min. afin de culoter les débris cellulaires. Le surnageant est ensuite cfg à 14000 X g pendant 30 min. Les culots sont lavés 1 X dans du PBS. Après la dernière centrifugation, les culots sont resuspendus dans un total de 6 ml de PBS (1 ml par boîte) et conservés à - 20 ° C. Cinq cycles de gel (- 20 ° C) - dégel (37 ° C) sont réalisés. Le matériel contenant les *Cowdria* tuées est conservé à - 20 ° C.

Un stock de *Cowdria* (Gardel) vivantes a également été préparé. Les *Cowdria* sont cultivées dans des BUEC de la même manière que celle décrite précédemment. Cependant, après la centrifugation à 14000 X g, les culots sont resuspendus dans du SPG et directement congelés dans l'azote liquide.

ANNEXE

Inhibitory Effect of Recombinant Bovine Interferon Gamma on the Growth of *Cowdria ruminantium* in Bovine Endothelial Cells In Vitro.

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INTRODUCTION

Cowdriosis (also called heartwater) is a tick - borne disease of wild and domestic ruminants caused by the obligate intracellular rickettsia *Cowdria ruminantium* (6) and present in sub - saharan Africa and in the french west Indies (27). Although a certain degree of innate resistance against cowdriosis exist among indigenous ruminants, the disease becomes a major problem when exotic breeds of ruminants are introduced (16). Also, risks of introduction of this disease onto the american mainland exist (26, 2), thus posing a serious threat for the american livestock.

Animals that survived the infection are immunized against homologous challenge but the mechanisms involved in protective immunity against *C. ruminantium* are totally unknown. Altough the role of antibodies should not be underestimated, preliminary studies suggest that the nature of protective immunity is largely cell - mediated (27). The fact that T lymphocytes of the Lyt-2^+ phenotype collected from immunized mice confer protection against *C. ruminantium* to susceptible mice (8) is supporting evidence. However, the exact mechanisms of protection induced by immune Lyt-2^+ cells (e.g. cytotoxicity for *Cowdria* - infected cells and release of cytokines) remain to be elucidated. We have shown recently (23) that IFNα, wich can be produced by activated T cells (11), is induced in cattle that naturally (without antibiotic treatment) resist an experimental infection with *C. ruminantium* . The growth of the pathogen in vitro in bovine vascular endothelial cells (one of the target of *C. ruminantium* in vivo) is significantly slowed down by recombinant bovine IFNα but is not totally blocked (23), suggesting that

other mechanisms are necessary for the control of the infection. A possible candidate is IFNγ, wich is also produced by activated T lymphocytes and has been shown to play an important role in the resistance against several other rickettsias (4). Recombinant IFNγ inhibits the in vitro growth of *Rickettsia prowazekii* (24), *Rickettsia tsutsugamushi* Gilliam strain (9), *Chlamydia psittaci* (3), *Chlamydia trachomatis* (19) and *Ehrlichia risticii* (15). In vivo, a protective role of endogenous IFNγ has been shown for *Rickettsia conorii* (14) and *Chlamydia trachomatis* (29, 17).

In this report, we show for the first time that recombinant bovine IFNγ completely prevents the multiplication of several stocks of *C. ruminantium* , in vitro, in bovine and goat vascular endothelial cells.

MATERIALS AND METHODS

Cell cultures. Two bovine and one goat endothelial cell lines were used in this study. Bovine endothelial cells from the microvasculature (BME) of brain adreno cortex were kindly provided by Dr. G. Tarone (University of Torino, Italy). Bovine endothelial cells from umbilical cord arteries were a kind gift of Dr. F. Jongejan (University of Utrecht, The Netherlands). Goat endothelial cells (CJE) were isolated from the jugular vein according to established procedures (18). These cells are positive for factor VIII production as shown by immunofluorescence (data not shown) indicating that they are of endothelial origin. All these cell lines are fully permissive to *C. ruminantium* growth. All cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10 % fetal calf serum (Gibco), penicillin (100 IU/ml), streptomycin (100 µg/ml) and

L-Glutamine (2mM).

Cowdria **stocks and in vitro culture.** Three different stocks of *C. ruminantium* were used in this study. One stock from Senegal (12), the Welgevonden stock from South Africa (7) and the Gardel stock from Guadeloupe (28). All these stocks were kept as stabilates from in vitro infected endothelial cells, and cryopreserved in sucrose - phosphate - glutamate (SPG) buffer (1). The culture of *C. ruminantium* in endothelial cells was done as previously described (22). Briefly, endothelial cells were grown to confluency in 75 cm^2 tissue culture flasks (Nunc). Cells were infected using SPG - cryopreserved stabilates of *C. ruminantium* diluted in "complete medium" composed of : Glasgow minimum essential medium (GMEM, Gibco) supplemented with tryptose phosphate broth (Gibco) at 2,9 g/l, penicillin (100 IU/ml), streptomycin (100 µg/ml); hepes (20 mM, pH 7.0 - 7.2), L-Glutamine (2 mM) and 10 % fetal calf serum (Gibco). The cells were incubated stationary in a 5 % CO_2 - 37 °C incubator. *C. ruminantium* replicates within intracytoplasmic vacuoles resulting in the formation of large colonies (morulae) that can be easily observed under a light microscope (inversed microscope, Leitz Diavert) or after staining of the cells with Giemsa or Diff-quick (Baxter).When more than 70 % of the cells were lysed by the rickettsia, the culture supernatant was centrifuged for 15 min. at 15000 X g. The pellet obtained from 2 ml of supernatant was resuspended in 1 ml SPG buffer before snap freezing in liquid nitrogen.

One - step growth - yield assay for ***C. ruminantium*** **.** We have studied the effect of recombinant bovine IFNγ on the infectious yield of *C. ruminantium* in endothelial cells infected in vitro. This was done by titrating the infectivity of supernatants from *Cowdria* - infected (in the

presence or absence of IFNγ) endothelial cells using a tissue culture lethal dose 50 % test (TCLD50) we have established (23). Endothelial cells were grown to confluency in 24 - well plates (Nunc) in complete medium (see *C. ruminantium* culture). The cells were infected with *Cowdria* - infected supernatant (collected from a culture showing 70 - 80 % cell lysis due to *C. ruminantium*) diluted two-fold in fresh complete medium (final volume: 1 ml per well). The cells (triplicate wells) were treated with medium alone (control) or with various concentrations of IFNγ at different time intervalls (the medium was replaced 24 h after each treatment). In order to confirm the involvment of IFNγ, the experiment was repeated in the presence of neutralising antibodies for bovine IFNγ. The progress of the infection was followed daily by light microscopy. When control wells (infected but not treated) showed 70 - 80 % lysis due to the rickettsia, all supernatants were collected. Each supernatant was centrifuged at 15000 X g for 15 min. and the pellet was resuspended in 100 µl SPG before snap freezing in liquid nitrogen. The infectivity of these supernatants was measured by a TCLD50 assay adapted for *C. ruminantium* (23). Results are expressed in % inhibition of *Cowdria* yield (± standard deviation) compared to the control.

IFN, IFN assay, and anti-IFN antibodies. Recombinant Bovine Interferon gamma (rBoIFNγ, specific activity 2.10^6 U/mg) produced in E. Coli and neutralizing antibodies to BoIFNγ were kindly donated by Dr. R. Steiger from CIBA - GEIGY. The titer of rBoIFNγ was regularly measured but the classical test of reduction of the cytopathogenicity of vesicular stomatitis virus (21) on Madin Darby bovine kidney cells (MDBK). The antiviral activity is expressed in laboratory units, no International Standards being available as reference for bovine IFNs. One unit of

antiviral activity is defined as the reciprocal of the dilution that gives 50% protection against viral challenge under standard conditions. In our test, a 10 fold dilution of anti - BoIFNγ antibodies was shown to neutralize 1.10^3 U of rBoIFNγ.

RESULTS

The effect of rBoIFNγ on the yield of *C. ruminantium* infectious particles in vascular endothelial cells was studied in vitro. rBoIFNγ reduced the yield of infectious *C. ruminantium* (stock Senegal) in BME cells in a dose - dependent manner (Fig.1). Although pretreatment of BME cells with rBoIFNγ, 24 h. prior to infection, provoked a certain degree of inhibition of *Cowdria* - growth, rBoIFNγ was more effective when added after adsorption of *C. ruminantium* (e.g., 24 h. after infection; Fig.1). In these conditions, 100 % inhibition of *C. ruminantium* yield was achieved with as litle as 0,5 U/ml of rBoIFNγ. The inhibitory effect was completely reversed by addition of anti - rBoIFNγ antibodies to the medium (Fig.1). On the contrary, addition of 100 U/ml of polymixin B (a known chelator of endotoxins) did not affect the inhibition of *Cowdria* yield by rBoIFNγ (not shown). It should be noted that although 100 % inhibition of the *Cowdria* infectious yield was observed 10 days post - infection, when cells were treated with 1 U/ml of rBoIFNγ, colonies of *C. ruminantium* were still visible in the cytoplasm of BME cells (Fig.2 B). On the other hand, when the cells were treated with 10 U/ml of rBoIFNγ at days 0 and 1, no more colonies of *C. ruminantium* were observed in these cells for up to 30 days post - infection (Fig.2 C). Re-infection was not tested. Cytotoxicity of rBoIFNγ for uninfected or *C. ruminantium*-

infected BME and BUE cells was observed but only when 50 U/ml or more were added to the medium for three consecutive days.

As shown in fig. 3, bovine endothelial cells are sensitive to the anticowdria effect of rBoIFNγ independantly of their origin. Indeed, microvasculature (BME) or macrovasculature (BUE) endothelial cells were equally rendered non-permissive to *C. ruminantium* replication by rBoIFNγ (Fig.3). Goat endothelial cells from jugular veins (CJE) were also sensitive to the anticowdria effect of rBoIFNγ (Fig.3). The apparent reduced sensitivity of CJE compared to BME and BUE cells could be explained by a species preference of rBoIFNγ.

The growth of three stocks of *C. ruminantium* originating from different parts of the world, was found to be efficiently inhibited by IFNγ in BUE cells (Fig.4). The experiment was done seperately for each stock. The *C. ruminantium* stocks used have been previously shown to be antigenically different in cross-immunity trials (13). Our results thus demonstrate an ubiquitous effect of rBoIFNγ on *C. ruminantium* growth in vitro which is dose-dependent.

DISCUSSION

In this report we show that rBoIFNγ is an excellent inhibitor of the infectious yield of *C. ruminantium* in vascular endothelial cells in vitro. The growth of *C. ruminantium* in these cells is significantly inhibited at very low concentrations of rBoIFNγ, in comparison to what has been shown for other rickettsias in other cells. For example, in our model, 10 U/ml of rBoIFNγ is capable of completely preventing the formation of *C. ruminantium* colonies, whereas 100 U/ml of human recombinant IFNγ

are required to prevent infection of human epithelial cells by *Chlamydia trachomatis* (19). This may reflect a difference between species but also between the type of cells used. We found (22) that bovine endothelial cells are 10 - 20 fold more sensitive to the antiviral activity of bovine IFNs compared to bovine epithelial cells. Our results also show that rBoIFNγ is a much more efficient inhibitor of *C. ruminantium* when compared to rBoIFNα. Indeed, a significant reduction (more than 50 %) of *C. ruminantium* infectious yield was obtained with as litle as 0,1 U/ml of rBoIFNγ, whereas more than 10 U/ml of rBoIFNα was necessary (23). This striking difference of antirickettsial activity between IFNγ and IFNα has been shown for other rickettsias (10, 20). Since the bovine endothelial cells we used have the same sensivity to the antiviral activity of both rBoIFNγ and rBoIFNα (unpublished data), our results indicate that the anticowdria effect of BoIFN can be dissociated from its antiviral activity.

It is also apparent from this study that rBoIFNγ acts on the host cells to render them unsuitable for *C. ruminantium* replication rather than directly on the extracellular organism. The formation of *C. ruminantium* colonies is prevented in vascular endothelial cells treated with rBoIFNγ, but the exact stage of the developmental cycle of *C. ruminantium* (entry of the organisms in the cells, transformation of elementary bodies into reticulate bodies, or replication of reticulate bodies) on wich rBoIFNγ acts is not known. The inhibitory effect of rBoIFNγ on the infectious yield of *C. ruminantium* cannot be explained by specific lysis of infected cells (as it was shown for *Chlamydia trachomatis* , 5, *Rickettsia prowazekii*, 25, and *Rickettsia tsutsugamushi* , 9) since no cytotoxic effect was detected at concentrations of rBoIFNγ that completely prevented the formation of *C.*

ruminantium colonies in endothelial cells. Involvement of oxygen catabolism metabolites, tryptophan degradation, nitric oxide and receptors to trasferrine in the anticowdria effect of rBoIFNγ in vascular endothelial cells is currently under investigation in our laboratory.

Stock diferences in susceptibility to the inhibitory effect of IFNγ has been shown for *Rickettsia tsutsugamushi* (10), with certain strains being totally unaffected by IFNγ. Here, we show that three strains of *C. ruminantium* originating from different part of the world (Senegal, South Africa and Guadeloupe) and with different antigenic properties are fully susceptible to rBoIFNγ-mediated inhibition in vitro. Also, the growth of *C. ruminantium* is efficiently inhibited by rBoIFNγ in all types of endothelial cells we used (in vivo, *C. ruminantium* can be found in veins, arteries and cappilaries, 27), showing that this effect is not restricted to bovine but is also effective in a goat model of *C. ruminantium* infections in vitro.

Thus, rBoIFNγ appears as a very powerful inhibitor of *C. ruminantium* growth in vitro independently of the rickettsial stock, endothelial cell origin and ruminant specie. These results are in favor of an important role for IFNγ in the protective immunity against *C. ruminantium* infections.

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FIG.1: Inhibition of *C. ruminantium* (Senegal) infectious yield by rBoIFNγ in BME cells. rBoIFNγ was added to the medium at different concentrations and time intervals: 24 h. before infection (—△—); at day 0 (—▲—); at day 1 (—○—) and at days 0 and 1 (—□—). Some wells received rBoIFNγ at days 0 and 1 together with anti-rBoIFNγ (—■—). Supernatants were collected 9 days post-infection and their infectivity determined by the TCLD50 assay. The experiment was run in triplicate, bars indicate the standard deviation. Data are from one of four experiments.

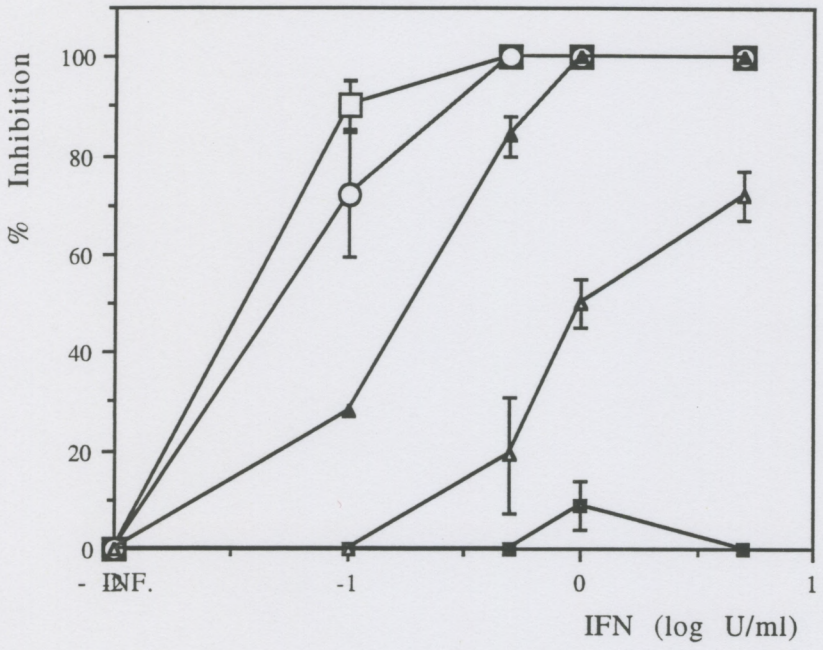


FIG.1

FIG.2: Inhibitory effect of rBoIFNγ on the growth of *C. ruminantium* (Senegal) in BME cells. Cells were rBoIFNγ treated at days 0 and 1 post-infection: 0 U/ml (A); 1 U/ml (B); 10 U/ml (C). The cells were stained (Diff-quick) 10 days post-infection (X 320; N: nucleus; C: colony of *C. ruminantium*).

N
C
C
C
N

FIG.3: rBoIFNγ-mediated inhibition of *C. ruminantium* (Senegal) infectious yield in different types of vascular endothelial cells . BME (—□—), BUE (—○—), and CJE (—◆—) cells were treated with various concentrations of rBoIFNγ at day 0 and 1 post-infection. Data (expressed as mean values ± standard deviation of triplicates) are from one of two experiments.

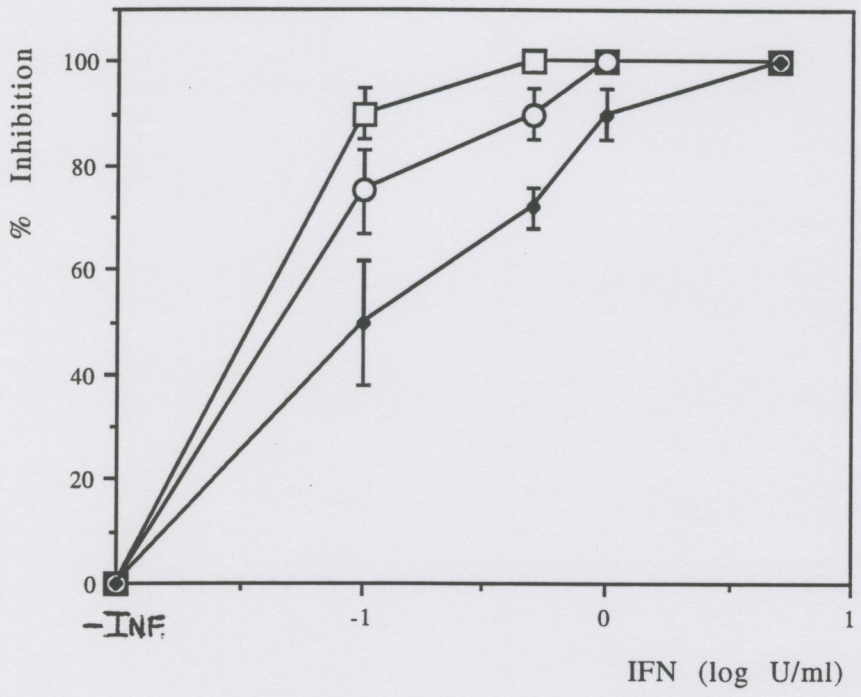
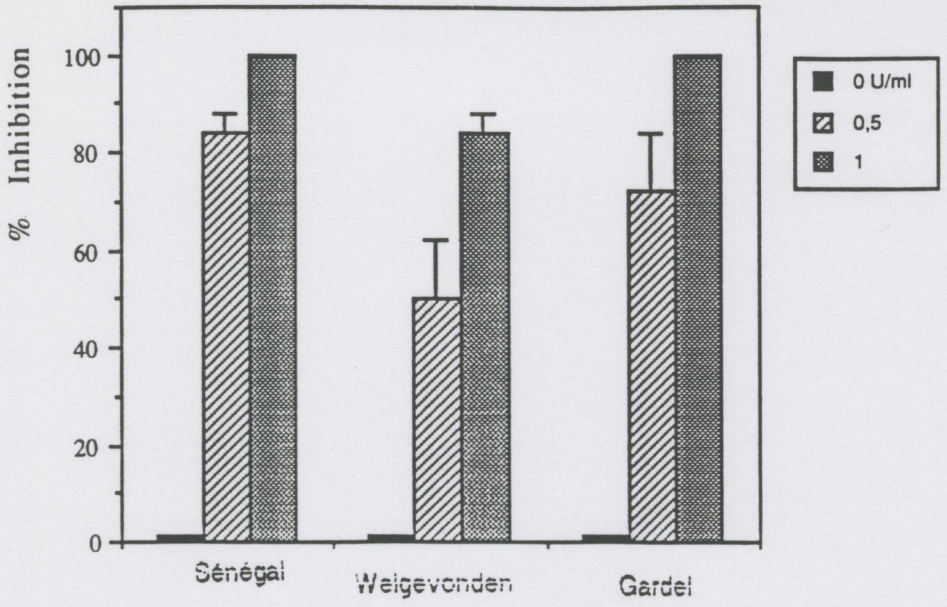


FIG. 3

FIG.4: Susceptibility of three different strains of *C. ruminantium* to the inhibitory effect of rBoIFNγ. BUE cells were treated with rBoIFNγ at day 0 and 1 post-infection. Data are expressed as mean values ± standard deviation of triplicates.


% Inhibition
100
80
60
40
20
0
Sénégal
Weigevonden
Gardel
0 U/ml
0,5
1